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Control of established experimental allergic encephalomyelitis by inhibition of tumor necrosis factor (TNF) activity within the central nervous system using monoclonal antibodies and TNF receptor-immunoglobulin fusion proteins

Tumor necrosis factor (TNF) activity was inhibited during the development of actively-induced, chronic relapsing experimental allergic encephalomyelitis (CREAE) in Biozzi AB/H mice, using a mouse TNF-specific (TN3.19.12) antibody and bivalent human p55 and p75 TNF receptor-immunoglobulin (TNFR-Ig) fusion proteins. The development of disease could be inhibited when repeated doses of antibody were administered prior to the anticipated onset. It has now also been shown that a therapeutic effect is evident even when antibody is administered after the onset of clinical signs, further indicating an important role for TNF in pathogenic effector mechanisms in CREAE. Although biologically-active TNF was not detected in the circulation, TNF-α was detected in lesions within the central nervous system (CNS). This suggested that the CNS may be the main site for TNF-specific immunomodulation and was supported by the observation that intracranial injection was significantly more potent than that administered systemically, for both antibody and TNFR-Ig fusion proteins. The fusion proteins were as effective as antibody at doses 10–100-fold lower than that used for antibody, reflecting their higher neutralizing capacity *in vitro*. Although treatment was not curative and relapse inevitably occurred in this model if treatment was not sustained, the data indicate that anti-TNF immunotherapy, especially within the CNS, can inhibit CREAE and may, therefore, be useful in the control of human neuroimmunological diseases.

1 Introduction

Tumor necrosis factor (TNF-α) and lymphotoxin (TNF-β) are cytokines with pleiotropic effects which may be necessary for optimal immune responses to tissue injury and infectious microorganisms. However, there is increasing evidence that TNF-α is involved in certain disease processes such as septic shock and arthritis [1–4]. The importance of TNF-α in these conditions have chiefly been established using neutralizing Ab [3, 4]. The numerous biological effects of TNF-α/β [1–2] are mediated following interactions with two receptors TNFR1 (p55) and TNFR2 (p75) [2]. Naturally occurring TNF inhibitors have been detected, which consist of the extracellular domains of the p55 and p75 TNFR [2] and provide candidates for immunoregulation of TNF. However, these monovalent proteins inhibit TNF activity only when present at a relatively high molar excess [5]. Fusion of these receptors onto Ig heavy chain constant regions exhibits advantages of a longer serum half-life and the ability to activate effector mechanisms via the Fc domain. In addition, the bivalency of the dimeric fusion proteins results in greater affinity for TNF, and a higher TNF-neutralizing capacity *in vitro* than their monovalent counterparts both for human TNF-α and TNF-β and mouse TNF-α [5–7] and may, thus, have clinical utility.

Chronic relapsing EAE (CREAE) is an autoimmune disease of the central nervous system (CNS) which is used as an experimental model for multiple sclerosis (MS). There is increasing evidence implicating a role for TNF in the pathogenesis of MS and EAE. Most encephalitogenic T cells secrete TNF following stimulation with myelin [8, 9] and T cells isolated from the CNS during active EAE [10] or from the cerebrospinal fluid (CSF) of patients with active MS exhibit a greater capacity to secrete TNF compared with T cells isolated from the peripheral blood during active disease or the CSF of clinically-inactive patients [11, 12]. TNF has also been identified in active EAE [13] and MS lesions [14, 15], where it could contribute to chronic damage associated with reactive gliosis and demyelination by the destruction of oligodendrocytes and myelin [16, 17]. While administration of exogenous TNF-α augmented actively induced EAE [18], initial studies failed to demonstrate any modulatory effect of polyclonal TNF-specific Ab on actively-induced EAE [19]. It is possible that actively induced disease may be less responsive to disease manipu-

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Abbreviations: **CSF:** Cerebrospinal fluid **CREAE:** Chronic relapsing EAE **i.c.:** Intracranial **MS:** Multiple sclerosis **p.i.:** Post-inoculation **SCH:** Spinal cord homogeneate

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don than adoptively transferred disease [20]. Monoclonal and polyclonal TNF-specific Ab significantly inhibited disease progression when administered to recipients of myelin-reactive T cell prior to disease onset [21, 22].

To investigate further whether TNF is important in the pathogenesis of neuroimmunological disease, TNF-specific mAb (TN3.19.12) [23] and TNFR-Ig fusion proteins were administered during actively-induced EAE. Treatments were given shortly before and during active clinical disease, when neurological signs were manifest. Both TNF-specific mAb and TNFR-Ig fusion proteins inhibited the development of the effector phase of CREAE, by targeting local production within the CNS.

2 Materials and methods

2.1 Animals

Inbred Biozzi AB/H ($H\text{-}2^{dq1}$) mice were bred at The Royal College of Surgeons of England and fed on RM-1(E) diet and water *ad libitum*.

2.2 TNF-specific reagents for *in vivo* treatment

TN3.19.12, a hamster IgG1 mAb which neutralizes mouse TNF-α and TNF-β [23] and L2 3D9, a non-neutralizing hamster IgG1 mAb reactive with mouse IL-2 which was used as a control, were both generously supplied by Dr. R. Schreiber (Washington University Medical School, St. Louis, MO) in conjunction with Celltech (Slough, GB). Chimeric antibodies expressing variable regions of TN3.19.12 and mouse IgG1 constant domains (cTN3.19.12.γ1) and mouse IgG1 mAb (MOPC 21) were supplied by Dr. M. Bodmer and Dr. A. Morgan at Celltech. Bivalent TNFR-Ig proteins were prepared by fusion of the extracellular domains of the human p55 and p75 TNFR to a partial human J sequence followed by all three constant regions of human IgG1 heavy chain, which was itself associated with the constant region of a truncated, κ light chain (B.J. Scallon, H. Trinh, Brennan F.M., Feldmann M. and Ghrayeb J., Comparison of the ability of different tumor necrosis factor receptor Fc fusion proteins to inhibit tumor necrosis factor; submitted for publication). The p75P-TNFR contained a deletion of the C-terminal 53 amino acids of the extracellular domain of p75-TNFR and exhibited comparable neutralizing capacity of human TNF-α as the p55-TNFR *in vitro* (Scallon; unpublished). This was stored at − 20 °C prior to use. Requests for available TNFR-fusions proteins should be addressed to B.J. Scallon, Centocor. The YTS-177.9 hybridoma was supplied by Prof. H. Waldmann, Sir William Dunn School of Pathology, Oxford, GB. YTS.177.9 which is a non-depleting mouse CD4-specific antibody [24], was produced in ascites fluid as described previously [25].

2.3 Induction of EAE

Biozzi AB/H mice were injected with 1 mg of spinal cord homogenate (SCH) emulsified with Freund's incomplete adjuvant supplemented with 60 μg mycobacteria (*Mycobacteria tuberculosis H37Ra* and *M. butyricum* [8:1]) on day 0 and 7 as described previously [25]. From day 11 (D11) post-inoculation (p.i.) onwards the mice were weighed and checked for clinical signs. These signs were graded as follows: 0 = normal, 1 = totally limp tail, 2 = impaired righting reflex, 3 = partial hindlimb paralysis and 4 = complete hindlimb paralysis. Neurological signs of lower severity than typically observed were scored 0.5 lower than the grade indicated [25]. Spinal cords and brains were removed, processed for routine histology and sections stained with hematoxylin and eosin.

2.4 Detection of TNF activity

2.4.1 Tissue fluids

Serum samples were prepared following exsanguination into the thoracic cavity, of terminally anesthetized animals during various phases of EAE. CSF samples (1–3 μl/animal) were withdrawn from the *foramen magnum* into a hematocrit tube. Following centrifugation to remove cells, these samples were stored at − 20 °C prior to assay. TNF activity was assessed using either the TNF-sensitive mouse fibroblast cell line L929, as described previously [26], or 1:2 dilutions of serum, and 1:50 dilutions of CSF were assayed using the Factor-Test mouse TNF-α ELISA kit (Genzyme, GB), according to the manufacturer's instructions. The ELISA assay detected 50 pg/ml − 3.2 ng/ml of TNF-α.

2.4.2 Tissue sections

Acetone-fixed cryostat sections of cervical CREAE spinal cord were stained within 1 week of preparation, by an indirect, avidin:biotin, immunoperoxidase technique essentially as described previously [13, 25], using TN3 19.12 mAb or rat anti-mouse TNF-α mAb (MP6-XT3 [HB10649] or MX6-XT22 [HB10697]) [27] obtained from the American Tissue Culture Collection, courtesy of Dr. J. Abrams (DNAX, USA). TN3 19.12 and MX6-XT22 failed to give satisfactory staining throughout a range of doses. However, 20 μl (4–8 μg/ml) of MX6-XT3 revealed staining which was inhibited when the primary mAb was diluted with excess recombinant mouse TNF-α (2000–500 μg/ml) prior to use for immunocytochemistry (not shown). This process failed to inhibit the staining of sections with a CD8a-specific mAb. For double labeling, sections were incubated with rat MX6-XT3; 1:100 dilution of rabbit anti-rat Ig (BA-4001. Vector) in PBS containing 5% normal mouse serum (NMS). This was used as a link reagent to increase the sensitivity of the staining, and a 1:100 dilution, in 5% NMS, of swine anti-rabbit Ig which was conjugated with either TRITC (R156. Dako) or FITC (F205. Dako). Rabbit anti-factor VIII-related antigen (A082. Dako) and rabbit anti-glial fibrillary protein (GFAP. Z334. Dako) were conjugated with FITC and extensively dialyzed. Double immunofluorescence staining was then performed by incubating these sections with 1:50 − 1:100 dilutions (in 5% NMS) of either: FITC-conjugated anti-factor VIII, anti-GFAP, H-2A (Ia. 17)-specific mouse mAb (OX-6. Serotec, GB), or PE-conjugated rat Ig mAb specific for B cell-restricted B220, CD4 or CD8 antigens (Coulter Ltd, GB) for 30 min. Sections were observed by fluorescence microscopy.

2.5 *In vivo* mAb and receptor treatment

Antibodies and receptors were administered to SCH-immunized EAE animals at various times post-inoculation. Mice were typically injected i.p. with 0.1 ml of mAb diluted in PBS. In some instances anesthetized mice received a single 30–40 µl i.p. or intracranial (i.c.) injection in the cortex of the right frontal lobe as previously described [25].

2.6 Oxazolone proliferative assay

Of 2.5 % oxazolone (OX, Sigma, GB) 25 µl dissolved in 4:1 acetone:olive oil (AOO) was painted on one ear on day 0 [25]. On day 2 animals were injected i.p. with 0.1 ml of TNF and CD4-specific mAb. Three days after the topical application of oxazolone the draining auricular lymph nodes from three-four animals/group were removed and pooled, and the induced proliferative response assessed as previously described [25]. Briefly, 5×10^5 cells/well were cultured overnight (in the absence of exogenous oxazolone) at 37 °C in a humidified atmosphere of 5 % CO_2 in air with 2 µCi of [methyl-^{3}H] thymidine (sp. act. 2 Ci/mmol; Amersham, GB). Cultures were harvested and [^{3}H] thymidine incorporation was determined by β scintillation counting.

2.7 Inhibition of TNF cytotoxicity by TNF-specific reagents

The mouse fibrosarcoma cell line WEHI 164 [28] was seeded in 96-well tissue culture plates at 2×10^4 cells/well in 100 µl DMEM supplemented with 5 % FCS, 0.04 M sodium bicarbonate, 2 mM L-glutamine, 100 U/ml of penicillin and 100 µg/ml streptomycin and allowed to adhere overnight at 37 °C. A suboptimal concentration of mouse TNF-α (15 pg/ml), sufficient to generate 70 % cytotoxicity which was within the linear region of the dose-response curve was preincubated with serial dilutions of p55, p75P TNFR-Ig fusion proteins and TN3.19.12 mAb for 1 h at 37 °C and 100 µl/well were added to the cell cultures with 10 µl of 10 µg/ml actinomycin D. After 24 h of culture 10 µl MMT, (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) at 5 mg/ml in PBS, was added followed by an overnight incubation at 37 °C. Formazan crystals were then dissolved by the addition of 100 µl of 10 % sodium dodecyl sulfate in 0.01 M HCl per well and further incubated overnight at 37 °C. The absorbance of cultures was assessed at 590 nm.

2.8 Statistical analysis

Statistical analysis between groups was performed using Wilcoxon's nonparametric test.

3 Results

3.1 Systemic anti-TNF immunotherapy

To elucidate the potential role of TNF in a chronic relapsing EAE model, animals were injected with TN3.19.12, a neutralizing TNF-specific mAb with a serum half-life of approximately 7 days [23]. A single injection of 300 µg of TN3.19.12 has been reported to inhibit the development of relapsing EAE induced by cell-transfer [21]. However, in this study, a single i.p. injection of 250 µg of TN3.19.12 on day 12 post-inoculation following active sensitization failed to prevent animals (7/8) developing EAE compared to those (8/8) injected with the control (L2 3D9) hamster Ig mAb. However, this TN3.19.12 treatment appeared to ameliorate the severity of disease. In comparison to L2 3D9-treated animals, mice injected with TN3.19.12 appeared to exhibit a delayed onset of weight loss (day 16.1 ± 2.5 *vs.* 14.0 ± 0.9) and clinical signs (day 17.0 ± 2.0 *vs.* 15.4 ± 1.2) and a lower severity of maximum clinical signs (2.1 ± 1.1 *vs.* 3.1 ± 0.9) and body weight loss (25.5 ± 4.6 % *vs.* 29.0 ± 4.8 %), but these failed to reach statistical significance. Furthermore, animals which developed clinical disease following TN3.19.12 treatment, subsequently relapsed with 6/7 animals relapsing (day of onset 38.3 ± 7.5) compared with that observed in controls where 5/6 animals relapsed on day 39.6 ± 5.3 when observed to day 55 post-inoculation.

It appeared that TN3.19.12 mAb administered just prior to the onset of clinical manifestations may inhibit the onset of EAE by 2–3 days. Therefore, the animals were given multiple i.p. (250 µg) Ab doses, at 3-day intervals, initiated prior to and during the anticipated development of weight loss (> 1.5 g/day), which occurs 1–2 days before the onset

Table 1. Multiple doses of systemically administered TNF-specific mAb inhibits the development of experimental allergic encephalomyelitis[a)]

Treatment	Results up to day 23 p.i.		Results up to day 30 p.i.		
	No. EAE/total	Clinical score	No. EAE/total	Clinical score	Day of onset
PBS	13/15	2.6 ± 0.4	15/15	2.7 ± 0.4	17.7 ± 0.7
Hamster Ig (L2 3D9)	11/16	1.7 ± 0.4[b)]	14/16	1.8 ± 0.4	18.4 ± 1.4[d)]
anti-TNF (TN3.19.12)	4/16[b)]	0.7 ± 0.3	13/16	1.1 ± 0.3[b)]	22.5 ± 0.7[c)]

a) Animals were immunized with 1 mg SCH in Freund's adjuvant on day 0 and day 7. Mice were injected i.p. with 0.1 ml of purified mAb containing 250 µg of either L2 3D9 (control) or TN3.19.12 (TNF-specific) antibodies on day 14, 17 and 20, shortly before and during the onset of anticipated clinical disease. The results represent the number of animals which had developed disease, and the maximum clinical grade exhibited within each group, three and ten days following the termination of antibody treatment.
b) $p < 0.01$.
c) $p < 0.002$ compared with the PBS-treated group.
d) $p < 0.05$ compared with the TNF-i.p.-treated group.

of clinical signs [29]. In comparison to PBS and L2 3D9-treated controls, multiple doses of TNF-specific mAb significantly ($p < 0.002$ and $p < 0.05$, respectively) inhibited the development of EAE when assessed up to 3 days following the cessation of treatment (Table 1). Although only 4/16 animals experienced clinical EAE during this period, within 10 days of mAb treatment the majority (13/16) of the animals subsequently developed clinical signs (Table 1). This represented a significant delay ($p < 0.002$) in the onset of clinical signs (Table 1). Treatment with L2 3D9 (a non-neutralizing anti-IL-2 mAb) appeared to reduce the severity of clinical signs expressed, but was not significantly different from PBS-injected animals.

In all instances in this study, clinical outcome was the primary assessment, therefore clinical signs were allowed to remit. During EAE in the AB/H mouse the extent of mononuclear cell infiltration correlates with the severity of clinical disease and abates rapidly during clinical remission [29]. Although anti-TNF-treated animals failed to show much evidence of histological lesions, this was also the case in untreated EAE-remission animals and the histology was therefore not particularly informative.

3.2 Detection of TNF in the CNS during EAE

Following the induction of EAE clinically-normal animals exhibited weight loss, which progressed as paralysis developed (25–35% body weight compared with D11) and correlated with development of blood:brain barrier dysfunction and cellular infiltration [29, 30]. However, initial examination of sera, with the L929 cell line, from animals exhibiting either weight loss alone, prior to the onset of signs [29], or paralysis, failed to demonstrate the presence of biologically active TNF. Furthermore, analysis of five individual samples of sera following weight loss, the onset of signs (limp tail), paralysis, and post-acute animals [29] and CSF samples from five paralyzed animals indicated that the level of TNF present was below the sensitivity of the TNF-α ELISA assay used. This suggests that at least in the serum samples, there was less than 100 pg/ml of biologically

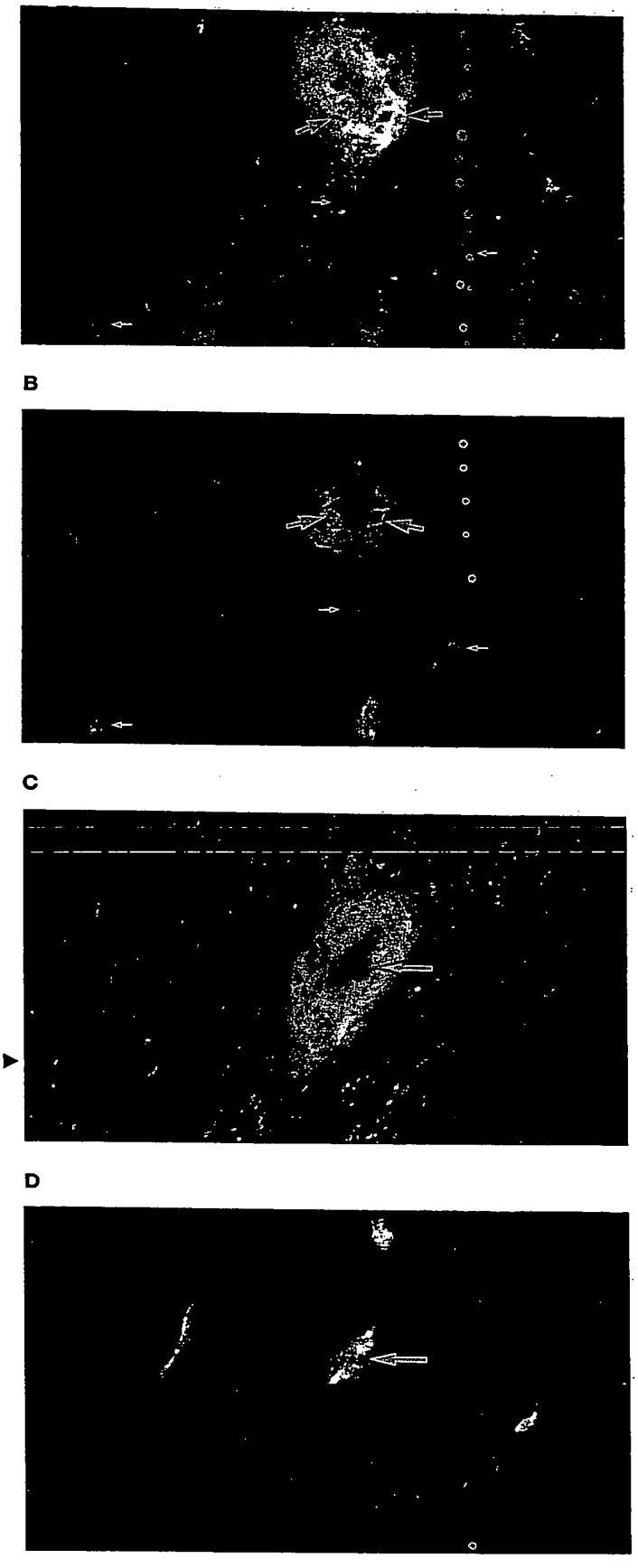
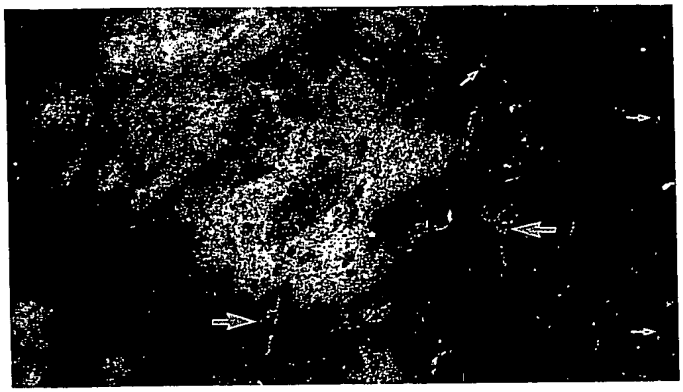


Figure 1. Immunofluorescence detection of TNF-α on CD4+ T lymphocytes, astrocytes and macrophages in spinal cord lesions during CREAE. TNF-α was detected by either FITC-(A) or TRITC-(C, E and G) conjugated Ab in spinal cord lesions of CREAE. Sections were then incubated with either a PE-conjugated CD4-specific mAb (Fig. 1B) or FITC-conjugated anti-Factor VIII related-antigen (D), anti-GFAP (Fig. F) or H-2A-specific antibodies (H). Following photography of CD4 antigen staining (B) the sections were exposed to sufficient ultra violet light to bleach the PE and thus, prevent interference of PE emission in the FITC channel. Although the majority of CD4+ T lymphocytes do not express detectable TNF-α activity (small arrow), ocassionally TNF-α activity co-localized with cells (arrow) expressing CD4 antigen (B). Endothelial cells (D), however, generally failed to co-express TNF-α, even within perivascular lesions (arrow). Although not all TNF activity co-localized with the expression of GFAP (F), astrocytes expressing TNF-α where readily detectable (arrow) and could be localized to part of the astrocytic processes (small arrow) surrounding lesions. This astrocytic staining profile of TNF-α can be observed in A, C and G. Furthermore, macrophages/microglia expressing MHC class II antigens (H) could readily be shown to express TNF-α (arrow) within CNS lesions during EAE. (× 400)

E



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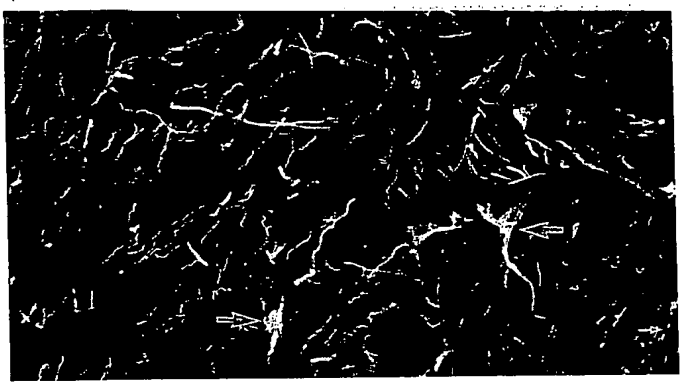

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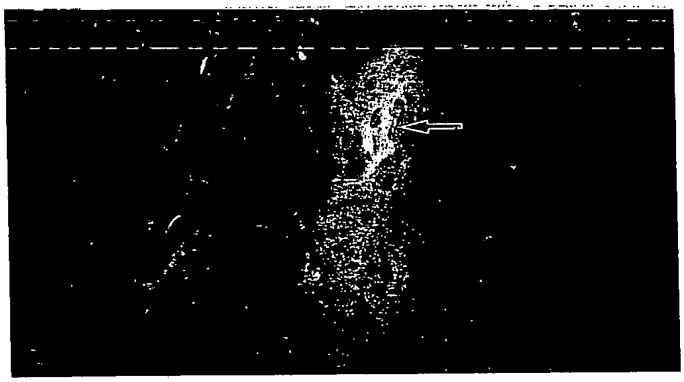

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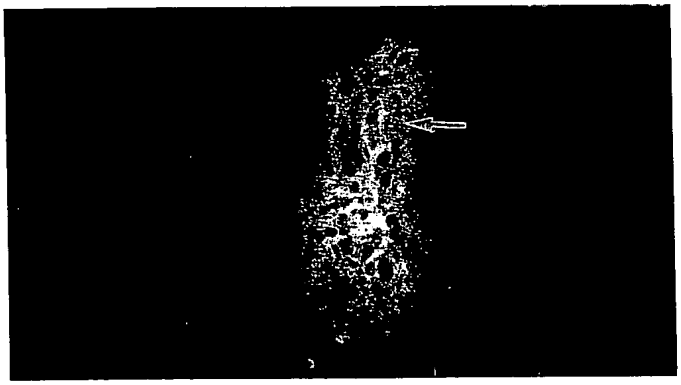

active TNF-α. However, it was possible to detect TNF-α within the CNS of EAE animals using immunocytochemistry, where staining probably represented a combination of soluble and membrane-bound TNF-α (Fig. 1). TNF-α was found in mononuclear cells within perivascular lesions and was often concentrated at the parenchyma/lesion edge, where positive cells appeared macrophage/glial-like. During the acute phase of CREAE both B cells and $CD8^+$ T lymphocytes form a minor component of the cellular infiltrate [29] and B cells generally failed to show any evidence of TNF-α by double immunofluorescence staining. Occasionally some $CD4^+$ T lymphocytes within perivascular lesions expressed TNF-α (Fig. 1A and 1B). Although TNF was detected in close proximity to blood vessels, staining typically failed to co-localize with endothelial cells stained by anti-Factor VIII-related antigen (Fig. 1C and 1D). While some $GFAP^+$ astrocytes expressed TNF-α (Fig. 1E and 1F) particularly in areas adjacent to perivascular lesions, the majority of detectable TNF-α activity co-localized with macrophage/microglia expressing MHC class II antigens (Fig. 1G and 1H).

3.3 Systemic anti-TNF immunotherapy after the onset of clinical disease

Having established that TNF was present within lesions (Fig. 1), animals were injected with TNF-specific mAb when clinical signs were first manifest; that is when the

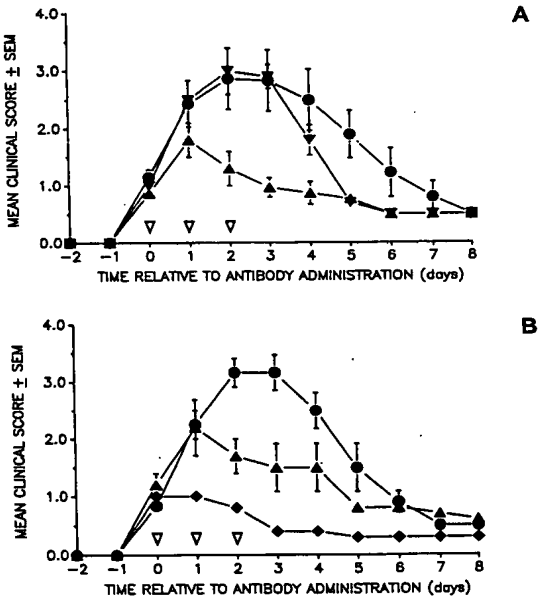

Figure 2. Inhibition of the development of clinical disease following injection of TNF-specific mAb. Animals were injected with SCH in Freund's adjuvant on day 0 and 7. Following the onset of clinical signs, when animals were exhibiting a flaccid tail (day 0), mice were injected (arrows) i.p. with 0.1 ml of either PBS (circles), 250 μg (A) or 1 mg (B) of TNF-specific antibody (triangles), or 250 μg hamster Ig (L2 3D9; inverse triangles) on day 0, 1 and 2 following the onset of signs. Furthermore, animals received a single injection of a CD4-specific non-depleting antibody (YTS 177.9; diamonds in B only), approximately 250 μg [25] on day 0 only. The results represent the mean group clinical score ± SEM ($n = 5–7$).

animals were exhibiting a flaccid tail (grade 1). Following the first injection of TN3.19.12 antibody the clinical signs progressed (Fig. 2), and antibody treatment was, therefore, continued daily for an additional 2 days. Within 2 days of administering 250 μg of TN3.19.12 clinical signs abated and were significantly different ($p < 0.02$) from L2 3D9-treated animals, whose disease became more severe. Although not statistically significant, L2 3D9-treated animals appeared to remit at a faster rate than PBS-treated animals. This suggested that this non-neutralizing IL-2-specific antibody may be exhibiting some biological inhibitory effect (Fig. 2A). Increasing the dose of TN3.19.12 to 1 mg failed to improve the inhibitory effect above that observed with 250 μg TN3.19.12 (Fig. 2B). As has previously been shown [25], a non-depleting CD4-specific mAb could rapidly stabilize and reverse clinical progression (Fig. 2B). Although the mechanisms by which these antibodies act remain to be established, the observation that TNF-specific immunotherapy failed to inhibit an *in vivo* induced proliferative response whereas anti-CD4 treatment was markedly immunosuppressive (Fig. 3), suggests that these mechanisms are different.

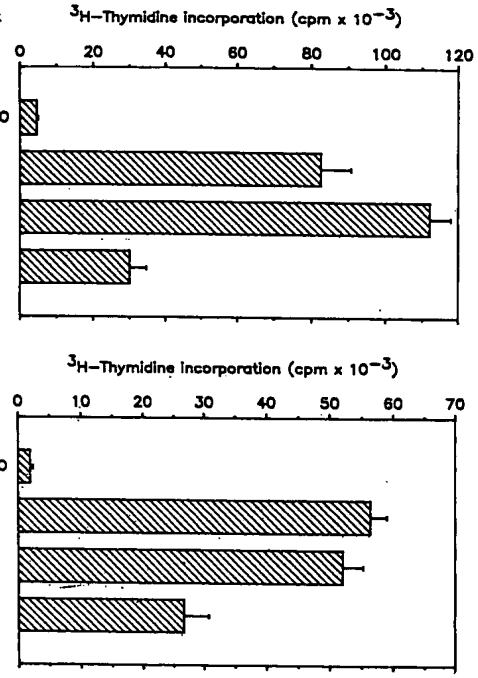

Figure 3. Effect of TNF immunotherapy on an *in vivo* induced T-cell proliferative response. Animals were painted on the ear with 25 μl of 2.5% oxazolone on day 0. Two days later animals (three-four per group) were injected ip. with 500 μg TNF-specific antibody (TN3.19.12) or approximately 250 μg CD4-non-depleting antibody (YTS 177.9). Draining lymph nodes cells (5×10^5) were removed and pooled and cultured in the presence of [^{3}H] thymidine overnight. The results represent the mean ± SD of a minimum of five replicate wells. The results of two individual experiments are shown.

3.4. CNS-directed TNF immunotherapy

As shown in Fig. 2B it is possible to stabilize rapidly and reverse disease progression with CD4-specific mAb. Although CD4+ cells can be targeted in the peripheral circulation prior to extravasation into the CNS, it is probable that the majority of the TNF activity/secretion is occurring within the CNS (Fig. 1), and efficient immunotherapy may therefore require sufficient antibody to enter the CNS. To elucidate this further, different routes of administration of TN3.19.12 were examined. Following the onset of clinical signs animals were injected intracranially with varying doses of TN3.19.12 (Fig. 4). Although 1.5 μg of TN3.19.12 failed to alter the clinical course of disease, 150 μg of mAb stabilized clinical disease (Fig. 4). The systemic i.p. injection of clinically-affected animals with 150 μg of mAb TN3.19.12 again initially failed to prevent rapidly the progression of disease (Fig. 5). However, significant ($p < 0.002$) benefit was observed when TNF immunotherapy (150 μg mAb) was administered directly into the CNS (Fig. 5), compared to that administered systemically. In contrast to controls and animals treated

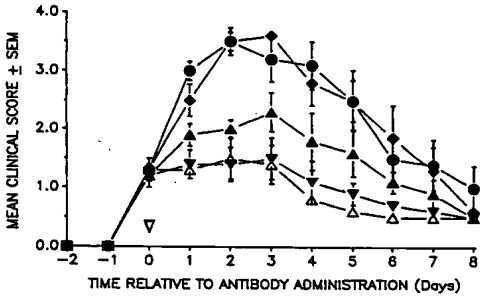

Figure 4. Dose-dependent inhibition of the progression of clinical EAE following injection of TNF-specific mAb directly into the CNS. Animals were injected with SCH in Freund's adjuvant on day 0 and 7. Following the onset of clinical signs (arrow), when animals were exhibiting a flaccid tail (day 0), mice were injected i.c. either with 30 μl of PBS (circles) or 30 μl of 5 mg/ml (150 μg; inverse triangles), 0.5 mg/ml (15 μg; triangles) or 0.05 mg/ml (1.5 μg; diamonds) of the TN3.19.12 mAb or 15 μg of p55-TNFR-Ig fusion protein (open triangles). The results represent the mean group score ± SEM of five-six animals per group.

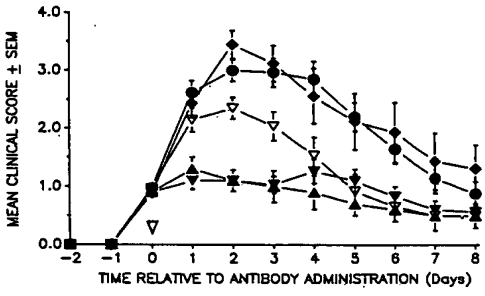

Figure 5. Inhibition of the development of clinical disease following injection of TNF-specific monoclonal antibody directly into the CNS. Animals were injected with SCH in Freund's adjuvant on day 0 and 7. Following the onset of clinical signs (arrow), when animals were exhibiting a flaccid tail (day 0), mice were either untreated (circles) or injected with 30 μl of PBS i.c. and 150 μg of TN3.19.12 mAb i.p. (open inverse triangles) or injected i.c. with 30 μl of 5 mg/ml TN3.19.12 mAb i.c. (150 μg) and PBS i.p. (closed inverse triangle). Alternatively, animals were injected i.c. with 150 μg of mouse IgG1 control (MOPC 21) mAb (diamonds) or chimeric TN3.19.12γ1 mAb (triangles). The results represent the mean group score of 5–16 animals per group.

systemically with TN3.19.12, where clinical signs always became more severe following disease onset, CNS-directed treatment generally stabilized clinical disease prior to remission, although in some cases animals experienced a transient increase in severity of signs following a period of stabilization. Furthermore, i.c. injection of TN3.19.12, into 18–19-g mice, significantly ($p < 0.002$) inhibited the progression of weight loss (0.08 g ± 0.21 g) within 24 h after treatment, compared to both untreated animals (1.37 ± 0.11 g) and mice i.p. injected with the TNF-specific antibody (1.00 ± 0.18 g). Although this treatment modulated the severity of clinical disease, the majority of both the controls (5/6) and anti-TNF i.c.-treated (5/6) animals subsequently relapsed on day 38.4 ± 3.4 and 35.6 ± 5.3, respectively. Because data presented in Table 1 and Fig. 2 suggest an ameliorative effect of the L2 3D9 hamster IgG1 mAb, mouse IgG1 (MOPC 21) was used as a non-specific control for the i.c. injection of mAb and failed to affect the course of disease compared with animals injected with PBS (Fig. 5). However, injection of chimeric TN3.19.12-γ1 mAb exhibited a comparable ameliorative effect to the parent TN3.19.12 hamster IgG1 mAb (Fig. 5).

3.5 Immunotherapy with soluble TNF-receptors

The p55 and p75P TNFR-Ig fusion proteins exhibited a significantly higher inhibitory capacity than the TN3.19.12 mAb in assays measuring mouse TNF-α-mediated killing of WEHI-164 cells *in vitro* (Fig. 6). This difference was further examined *in vivo* following i.c. treatment with TNFR-Ig fusion proteins. Of p55-TNFR-Ig 15 μg was found to induce an inhibitory effect comparable to 150 μg of TN3.19.12 mAb (Fig. 4), although as previously found with mAb treatment the animals treated with p55-TNFR-Ig subsequently relapsed (5/6 on day 30.6 ± 0.5 post-inoculation). Both p55 and p75P-TNFR-Ig were effective ($p < 0.002$) in disease inhibition (Fig. 7a). Further studies with p55-TNFR-Ig fusion protein indicated therapeutic effect at 1.5 μg following i.c. (Fig. 7a), which was comparable to that induced with 150 μg p55-TNFR-Ig fusion protein injected systemically (Fig. 7b). On a weight basis, both systemic (Fig. 7b *vs.* Fig. 2) and CNS (Figs. 4 and 7a *vs.* Fig. 4) administration of the TNFR-Ig fusion proteins exhibited a significantly improved inhibitory effect compared with TNF-specific mAb.

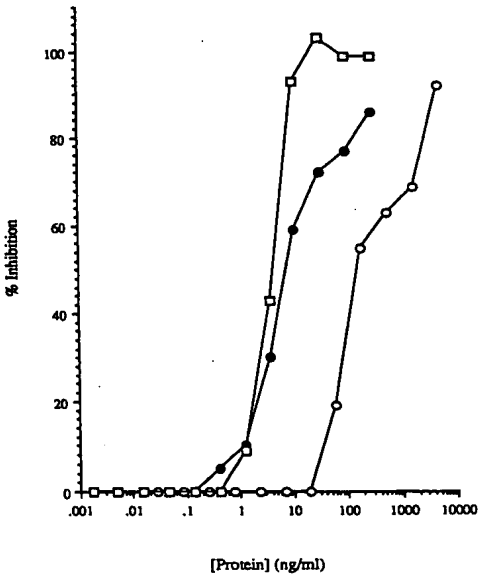

Figure 6. Inhibition of mouse TNF-α-induced cytotoxicity *in vitro* by TNFR-Ig fusion proteins. The TNF-sensitive WEHI 164 cells were incubated for 24 h with 15 pg/ml TNF, sufficient to generate 70% cytotoxicity, which was in the linear region of the dose-response curve in the presence of various concentrations of TN3.19.12 mAb (open cicles), p55-TNFR-Ig (open squares) or p75P-TNFR-Ig (circles) fusion proteins. Cytotoxicity was measured using the MTT colorimetric assay and reading absorbance at 590 nm; the percentage inhibition of cytotoxicity was calculated as follows:

$$\frac{A_{\text{sample + TNF}} - A_{\text{TNF alone}}}{A_{\text{sample}} - A_{\text{TNF alone}}} \times 100.$$

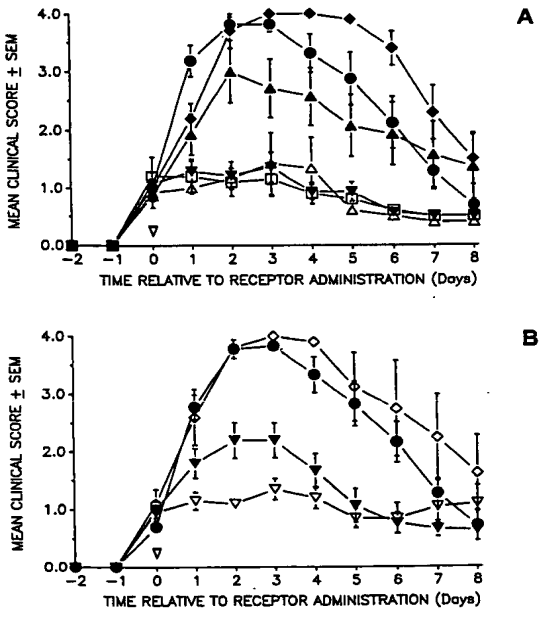

Figure 7. Inhibition of the development of clinical disease following injection of TNFR-Ig fusion proteins directly into the CNS (A) or systemically (B). Animals were injected with SCH in Freund's adjuvant on day 0 and 7. Following the onset of clinical signs (arrow), when animals were exhibiting a flaccid tail (day 0), mice were either untreated (circles) or injected i.c. (A) or (i.p.) B with either 150 μg (open diamond) or 15 μg (closed diamond) of an *in vitro* inactive p75-IgG1 fusion protein, as a control; 15 μg p75P-TNFR-Ig fusion protein (open square) or 150 μg (open inverse triangle), 15 μg (inverse triangle), 1.5 μg (open triangle) or 0.15 μg (closed triangle) of p55-TNFR-Ig fusion protein. The groups represent between 5–15 animals per group.

4 Discussion

This study demonstrated that neutralization of TNF *in vivo* with either TNF-specific mAb, p55 or p75-TNFR-Ig fusion proteins can significantly inhibit the development of actively-induced EAE in the mouse, even when clinical signs have already been established. Furthermore, increased inhibitory action on clinical progression can be observed when TNF immunotherapy is specifically directed into the CNS.

The development of clinical EAE induced by adoptive transfer has been prevented using both monoclonal [21] and polyclonal [22] Ab systemically administered to the recipients shortly after the transfer of encephalitogenic cells, prior to the development of clinical signs. It has been additionally demonstrated here that such treatment can inhibit clinical disease once it has been established, and implicates TNF as an important effector molecule during CREAE. In this CREAE model, induced in Biozzi AB/H mice by active sensitization, a single i.p. injection of a TNF-specific (TN3.19.12) mAb failed to prevent the development of relapsing disease, in contrast to the studies of relapsing EAE induced by cell transfer [21]. This result is similar to a previous study on actively-induced acute EAE in which benefit was not noted [19]. However, the development of EAE could be significantly inhibited if multiple doses of TNF-α/β-specific mAb were administered, as found previously in the treatment of cell-transferred disease, where daily administrations of a TNF-α-specific polyclonal antiserum were effective [22]. A recent report has found the incidence of relapse to be inhibited following repeated weekly administration of TN3.19.12 mAb to remission animals with CREAE induced by adoptive transfer [31]. In this and previous studies [22, 32] clinical EAE developed rapidly following the cessation of antibody therapy, and suggests that TNF immunotherapy is not exerting an effect through generalized, long-term immunosuppression. In contrast to the immunosuppressive action of CD4-specific mAb on EAE and T cell proliferation [25], inhibition of TNF activity exhibited minimal effects on T proliferative responses suggesting that TNF-directed immunotherapy targets effector cell function during chronic inflammation, rather than the induction of disease. This would be consistent with the inability of *in vitro* treatment of encephalitogenic cells to inhibit adoptive transfer of disease [22]. Therefore, the relative timing of antibody administration is important for an inhibitory effect to be observed. In contrast to the inhibitory effect of multiple doses observed when treatment was administered during the anticipated development of disease (Table 1), similar treatment (3 × 250 μg TN3.19.12 i.p.) terminated prior to development of anticipated clinical disease failed to prevent the development of clinical EAE (8/8 affected; mean group score 3.3 ± 0.5).

Although systemic administration of neutralizing TNF reagents inhibited EAE, a significantly increased inhibition of the progression of clinical signs and weight loss (data not shown) was observed when neutralizing TNF-specific mAb and TNFR-Ig fusion proteins were administered directly into the CNS. This suggests that an important component of TNF activity is generated within the CNS. This study indicates that the major source of TNF-α within the CNS during CREAE is from MHC class II-positive macrophages/microglia. However, it is also clear that T cells and astrocytes also express TNF-α during CREAE. In MS there is increasing evidence that TNF activity is concentrated within the CNS. Some studies have detected TNF in the serum of some but not all MS patient [11, 12]. In studies where TNF was measured in both peripheral blood and CSF, greater concentrations were detected in the CSF [11, 12]. TNF is present in CNS lesions [14, 15]. These reports showed TNF-α to be present in astrocytes and macrophages [14, 15], while TNF-β was expressed by T lymphocytes and microglial cells within the CNS white matter [15].

The precise mode of action of TNF-specific immunotherapy requires further elucidation, and the relative involvement of TNF-α and TNF-β in the neural tissue, during EAE and MS, is currently unknown. TNF-α produced locally within the CNS of nude mice has the effect of inducing acute, severe cachexia which is characterized by anorexia and weight loss, which contrasts with the slow, less-severe wasting disease induced following intramuscular implantation of cells producing TNF, even though serum levels of TNF-α were comparable to those obtained following i.c. implantation [33]. During active clinical EAE the blood : brain barrier (BBB) dysfunction correlates with the onset of weight loss [30] and in MS has been correlated with the levels of TNF-α [12]. Recently TNF-α has been shown to induce BBB breakdown [34] and lesions typical of EAE following direct injection into the CNS [35]. The data suggest that while TNF exhibits a variety of pro-inflammatory effects [1–2], it may particularly influence vascular permeability and leukocyte extravasation via up-regulation of leukocyte : endothelial adhesion molecules [1]. As such, treatment with TNF-specific mAb inhibits the up-regulation of adhesion molecules, expressed by the CNS vasculature, induced during adoptively transferred EAE [36] and prevents the accumulation of leukocytes along the entire neuroaxis [22]. TNF may also be involved in the development of active demyelination [17]. As cytokine expression appears to be developmentally regulated during lesion formation [37], it is likely that TNF may exert different effects depending on the constituents of the cytokine microenvironment present within the CNS.

Qualitatively the neutralization of TNF activity by TNFR-Ig fusion proteins was similar to that induced by mAb. Both could inhibit the development of CREAE while therapy was maintained. However the capacity to subsequently develop disease was not significantly altered, suggesting the TNF inhibitors acted by neutralizing locally-produced TNF rather than by directly inhibiting cytokine synthesis. Bivalent TNFR-Ig fusion proteins were significantly more effective than mAb at TNF neutralization *in vitro* and *in vivo* and supports previous studies using different TNFR-Ig fusion proteins in experimental models of toxic shock and sepsis [5–7]. This increased neutralization potential is probably mainly reflective of the increased affinity for TNF that the TNFR-Ig fusion proteins have ([5–7] and Scallon B.J., unpublished) compared to mAb, rather than differences in the Ig isotypes of the mAb and fusion proteins used. However selection of such Ig isotypes used in the neutralizing reagent may serve to influence the pharmacokinetics of bioavailability, and immunogeniticity of the TNF-neutralizing reagent. It seems clear from this study that cytokines produced within the target tissue are of major importance in the pathogenesis of disease. The CNS is normally relatively impermeable to Ig [25, 38]. However, it has previously been shown that i.c. administration can deliver significantly more Ab to the CNS, including the spinal cord, than can be delivered following systemic administration even during blood : brain barrier dysfunction which occurs during CREAE [25, 30]. The ability to use reagents of high neutralization capacity may be of particular relevance, when the ability of the reagent to enter

the target tissue is low, such as may occur in multiple sclerosis [38]. This may account for the increased efficacy of p55 TNFR-Ig fusion protein compared with mAb following systemic administration to inhibit the progression of disease development.

This and other studies have indicated that TNF is an important mediator in the pathogenesis of sepsis and importantly in autoimmune disease [3–5]. Studies in collagen type II arthritis in mice, with the same TNF-specific mAb (TN3.19.12) used in this current study, demonstrated an ameliorative effect on joint destruction [4], and this has led to a clinical trial of chimeric TNF-specific mAb in rheumatoid arthritis. That trial has confirmed the concept that TNF-α is a suitable therapeutic target in rheumatoid arthritis [39]. TNF is involved in the pathogenesis of CREAE, and on the available data appears to be a good candidate target for immune intervention in multiple sclerosis.

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